METHOD FOR DETECTING BINDING EVENTS USING MICRO-X-RAY FLUORESCENCE SPECTROMETRY

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STATEMENT REGARDING FEDERAL RIGHTS

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FIELD OF THE INVENTION

The present invention relates generally to binding events and more particularly, to a method for detecting binding events between receptors arrayed onto a substrate and at least one potential binder using micro-X-ray fluorescence spectroscopy.

BACKGROUND OF THE INVENTION

The desire to hasten the identification of potentially important polymers, drugs, catalysts, ceramic superconductors, phosphors, chemical and biological sensors, and other materials is a constant challenge that has prompted the use of combinatorial synthetic and screening strategies for synthesizing these materials and screening them for desirable properties. Combinatorial synthesis involves assembling a "library", i.e. a very large number of chemically related compounds and mixtures, usually in the form of an array on a substrate surface. Combinatorial screening involves identifying which members of the array, if any, have the desirable property or properties. The array form facilitates the identification of a particular material on the substrate.

The synthesis of a surface-bound array of oligopeptides, short chain products of the condensation of amino acids, has been described in PCT Publication No. WO 90/15070 to M. C. Pirrung et al. entitled "Very Large Scale Immobilized Peptide Synthesis", incorporated herein by reference. Oligopepeptides were chosen because they exhibit the types of binding specificity of their longer-chain polypeptide counterparts, such as proteins. The chemical

properties and in particular, the binding properties of a protein depend almost entirely on the exposed surface amino acid residues of the polypeptide chain. These residues can form weak noncovalent bonds with other molecules. An effective binding between the protein, one example of a group of materials herein referred to as "receptors", and the material that binds to the receptor, referred to herein as "binder", generally requires that many weak bonds form simultaneously between the protein receptor and the binder. Binders include organic molecules, inorganic molecules, salts, metal ions, and the like. The bonds between the protein and the binder form at the "binding site" of the protein. The binding site is usually a cavity in the protein that is formed by a specific arrangement of amino acids that often belong to widely separated regions of the polypeptide chain and represent only a minor fraction of the total number of amino acids present in the chain. Binders must fit precisely into the binding site for effective binding to occur. The shape of these binding sites can differ greatly among different proteins, and even among different conformations of the same protein. Even slightly different conformations of the same protein may differ greatly in their binding abilities. For further discussion of the structure and function of proteins, see: Bruce Alberts et al., "Molecular Biology of the Cell", 2nd edition, Garland Publishing, Inc., New York, 1989; and H. Lodish et al., "Molecular Cell Biology", 4th edition, W. H. Freeman and Company, 2000.

After a receptor array is prepared, it is screened to determine which members have the desirable property or properties. U. S. Patent 5,143,854 to M. C. Pirrung et al. entitled "Large Scale Photolithographic Solid Phase Synthesis of Polypeptides and Receptor Binding Screening Thereof", which issued September 1, 1992, hereby incorporated by reference, describes one such screening method. A polypeptide array is exposed to a ligand (an example of a binder) to determine which members of the array bind to the ligand. The ligands described are radioactive, or are "tagged", i.e. attached via one or more chemical bonds to a chemical portion that fluoresces when exposed to non-ionizing, ultraviolet radiation. Thus, the attached portion, i.e. the tag, makes the binder visible by

interrogation with ultraviolet radiation. Tagged molecules have also been used to aid in sequencing immobilized polypeptides as described, for example, in U. S. Patent 5,902,723 to W. J. Dower et al. entitled "Analysis of Surface Immobilized Polymers Utilizing Microfluorescence Detection," which issued May 11, 1999. Immobilized polypeptides are exposed to molecules labeled with fluorescent tags. The tagged molecules bind to the terminal monomer of a polypeptide, which is then cleaved and its identity determined. The process is repeated to determine the complete sequence of the polypeptide.

It is generally assumed that the attachment of a fluorescent tag to a potential binder only serves to make visible the otherwise invisible potential binder, and does not alter its binding properties. Since it is well known that even small changes to the structure of a molecule could affect its function, this assumption that a tagged binder, i.e. a "surrogate", has the same binding affinity as the untagged binder may not be a valid one. Small structural changes that accompany even a conformational change of a receptor have been known to affect the binding affinity of the receptor. The tagged surrogates are structurally different from their untagged counterparts, and these structural differences could affect their binding affinities. Since binding affinities derived using tagged surrogates are suspect, the binding properties of receptors and binders should be evaluated using the untagged binder or receptor and not with a tagged surrogate.

Therefore, an object of the present invention is to provide an efficient, combinatorial method of evaluating the binding properties of untagged potential binders with receptors.

Another object of the present invention is to provide a combinatorial screening method for directly comparing the binding properties of receptors/binders with their tagged surrogates.

Additional objects, advantages and novel features of the invention will be set forth in part in the description that follows, and in part will become apparent to those skilled in the art upon examination of the following or may be learned by practice of the invention. The objects and advantages of the invention may be

realized and attained by means of the instrumentalities and combinations particularly pointed out in the appended claims.

SUMMARY OF THE INVENTION

In accordance with the purposes of the present invention, as embodied and broadly described herein, the present invention includes a method for detecting a binding event between at least one binder and members of a receptor array. The method comprises the steps of exposing a plurality of receptors to at least one potential binder; arraying the receptors onto a substrate; exposing each member of the array to X-ray radiation; and detecting an X-ray fluorescent signal from any member of the array where a binding event has occurred.

DETAILED DESCRIPTION OF THE INVENTION

Briefly, the invention includes a method for detecting a binding event between members of a surface-bound receptor array and at least one potential binder using micro-X-ray fluorescence spectroscopy. A plurality of bead-supported receptors were exposed to at least one potential binder for a period of time sufficient for binding to occur, and then immobilized as an array onto a surface. Each member of the array was exposed to X-ray radiation. The detection of an X-ray fluorescence signal from a member of the array indicated that a binding event had occurred between that receptor and a binder. The intensity of the signal indicated the extent of the binding event, and could also be used to determine the binding affinity of the receptor to the binder.

The method of the invention uses X-ray fluorescence as a probe to detect binding events. X-ray fluorescence spectrometry is a powerful spectroscopic technique that has been used to determine the chemical elements that are present in a chemical sample, and to determine the quantity of those elements in the sample. The underlying physical principle of the method is that when an atom of a particular element is irradiated with X-ray radiation, the atom ejects a core electron such as a K shell electron. The resulting atom is in an excited state, and it can return to the ground state by replacing the ejected electron with an electron from a higher energy orbital. This is accompanied by the emission of a photon, i.e. X-ray

fluorescence, and the photon energy is equal to the difference in the energies of the two electrons. Each element has a characteristic set of orbital energies and therefore, a characteristic X-ray fluorescence spectrum.

An X-ray fluorescence spectrometer is an apparatus capable of irradiating a sample with an X-ray beam, detecting the X-ray fluorescence from the sample, and using the X-ray fluorescence to determine which elements are present in the sample and providing the quantity of these elements. The X-ray fluorescence spectrometer used to demonstrate the invention was the commercially available EDAX Eagle XPL energy dispersive X-ray fluorescence spectrometer, equipped with a microfocus X-ray tube, lithium drifted silicon solid state detector, processing electronics, and vendor supplied operating software. The EDAX Eagle XPL spectrometer can be used to determine the quantity in a sample of any element with an atomic number equal to or greater than the atomic number of sodium (sodium has atomic number 11). The vendor software used to operate the spectrometer allowed the simultaneous quantitation of 15 different elements for each member of the array.

The method of the present invention was demonstrated with a polymer bead-supported oligopeptide library purchased from Biopeptide Co., a commercial vendor. The library consisted of 625 unique 11-mer oligopeptides, i.e. each oligopeptide had a unique sequence of 11 amino acids. The oligopeptides used had the following general formula: NH_2-x-Gly-Gly-x-Gly-Gly-x-Gly-Gly-x-Phe-polymer. In this formula, "Phe-polymer" indicates that a phenylalanine amino acid of the oligopeptide is chemically bonded to the polymer bead support. The abbreviations for the amino acids in this formula are standard three-letter abbreviations used for the α-amino acids found in proteins and can be found in many textbooks (for example, see table 29.1 in F. A. Carey, Organic Chemistry, McGraw-Hill, 1987, pp. 1086-1087). In the formula: Phe is phenylalanine; Gly is glycine, NH_2 is the amine end group of the last amino acid of the chain, and x is any one of the following five amino acids: histidine (His); arginine (Arg); serine (Ser); tryptophan (Trp); and tyrosine (Tyr). Permutation of these amino acids

among the four 'x' positions generates a library of 625 unique 11-mer oligopeptides.

The bead support was a polymer made from Wang polystyrene resin. About 2 micrograms (μg) of each unique oligopeptide (corresponding to about 0.06 nanomoles, nmol) was supported on each bead. That is, each bead contains only one kind of 11-mer oligopeptide. The beads were about 80-120 micrometers in diameter, with an average diameter of about 100 micrometers.

The array format facilitated the identification of the particular oligopeptide-supported beads that exhibited the most significant X-ray fluorescence and therefore, the strongest binding effects. Once identified, these beads were then removed from the array and the amino acid sequence of the bound oligopeptides was determined by Edman degradation analysis. These oligopeptides were also characterized by MALDI-TOF MS, a mass spectroscopic technique. All other reagents used were commercially available and used without further purification.

The exposure of bead-supported receptors to potential binders such as organophosphates, organosulfonates, halides, metals, and the like, generally involved incubating the library of beads in an aqueous solution (pH 5.2-5.6) containing potential binder (about 0.5 – 100 μmol) for about 5-48 h at room temperature. In some cases, multiple treatments with a binder or binders resulted in an enhanced binding effect. After the incubation period, the beads were removed from the solution, washed with copious amounts of water, air-dried, and immobilized either on glass microscope slides with double-stick tape, or on tacky dot™ plates, to provide an oligopeptide receptor array. Tacky dot™ plates have arrays of adhesive dots on the surface and were used without any chemical modification. An X-ray fluorescence spectrometer was then used to determine which beads of the array had a measurable amount of binder attached. Each member of the array was exposed to spatially restricted X-ray radiation of a nominal beam size of 50 micrometers, and the X-ray fluorescence of particular elements were detected and quantified. If, for example, an organophosphate was tested as a potential binder, the element phosphorus was chosen as a detected

element. For potential organosulfonate binders, sulfur was chosen. For potential halide binders (as halogenated organic molecules), the particular halides (bromide for brominated organic compounds, chloride for chlorinated organic compounds, etc.) were chosen. For potential metal ion binders, the particular metal ion was chosen, etc. The X-ray fluorescence intensity was used to detect a binding event, and can also be used to determine the binding affinity of the binder to the receptor. The amount of receptor present on a bead can be verified from the X-ray fluorescence of the oligopeptide-supported bead prior to incubation in the binder solution. Quantification of the binder content was used to determine which of the substrate bound oligopeptide receptors were the most efficient receptors for binding a particular binder.

Particular examples of phosphates and phosphonic acids that were tested as potential binders included 4-chloro-5-bromo-indolyl phosphate, diethyl thiophosphate, diethyl dithiophosphate, diethyl cyanophosphonate, and methylphosphonic acid. Examples of organosulfates, sulfonic acids, and sulfides that were tested as binders include indolylsulfate, hydroxyethyl-piperazinepropanesulfonic acid, toluenesulfonic acid, L-cysteine, and N,N-diethylaminoethanethiol. Halide in the form of hydrogen chloride was also tested as a binder. Metal ions, in the form of soluble halide and nitrate salts, were also tested as binders; those tested include the following: $ZrCl_4$, $La(NO_3)_3$, $Ce(NO_3)_3$, $Pr(NO_3)_3$, $Nd(NO_3)_3$, $Sm(NO_3)_3$, $Eu(NO_3)_3$, $Gd(NO_3)_3$, $Tb(NO_3)_3$, $Dy(NO_3)_3$, $Ho(NO_3)_3$, $Er(NO_3)_3$, $Tm(NO_3)_3$, $Yb(NO_3)_3$, and $Lu(NO_3)_3$.

The method of the invention, in particular, was used to detect binding of N,N-diethylaminoethanethiol and/or methylphosphonic acid with members of the 11-mer oligopeptide library. An aqueous solution of these materials was prepared by combining 0.2 mL of a 200 mM solution of methylphosphonic acid (40 μmol) with 0.2 mL of a 210 mM solution of the hydrogen chloride salt of N,N-diethylaminoethanethiol (42 μmol). About 2500 of the oligopeptide-supported beads (corresponding to 5 mg total weight with about 1.6 μmol total oligopeptide) were incubated in the solution at room temperature for 2 days. The beads were

then removed from solution, washed with 10 mL of water, air dried, and immobilized on a tacky dot™ plate as an array. The beads of the array were then analyzed for binding using micro-X-ray fluorescence spectrometry using the EDAX micro-fluorescence instrument. Two beads in particular displayed a strong binding effect with methylphosphonic acid. Their amino acid sequences, as determined by Edman degradation analysis, were the following (the amine end group belongs to the amino acid at the end of the chain): 1) NH_2-His-Gly-Gly-His-Gly-Gly-His-Gly-Gly-Arg-Phe; and 2) NH_2-Tyr-Gly-Gly-Tyr-Gly-Gly-Trp-Gly-Gly-Tyr-Phe. Two different beads displayed a strong binding effect with the thiol. Their amino acid sequences, as determined by Edman degradation analysis, were the following: 3) NH_2-Ser-Gly-Gly-Arg-Gly-Gly-His-Gly-Gly-His-Phe; and 4) NH_2-Trp-Gly-Gly-His-Gly-Gly-His-Gly-Gly-Trp-Phe.

The invention was also used to screen potential metal catalyst binders. The chemical procedure employed was similar to one described by A. Berkessel and D. A. Herault in "Discovery of Peptide-Zirconium Complexes That Mediate Phosphate Hydrolysis by Batch Screening of a Combinatorial Undecapeptide Library", Angew. Chem.-Int. Ed. 1999, vol. 38, p. 102, hereby incorporated by reference. Berkessel et al. exposed a combinatorial array of bead-supported oligopeptides to zirconium tetrachloride. The resulting zirconium complex binds to some of the peptides, and some of the peptides with bound zirconium catalyze the hydrolytic cleavage of a phosphate ester. The catalytic activity of each exposed bead was determined by exposure to 4-chloro-5-bromo-indolyl phosphate, which undergoes phosphate ester cleavage to form 4-chloro-5-bromo-indoxyl and rapidly oxidizes to 4,4'-dichloro-5,5'-dibromo-indigo, an insoluble blue dye. The relative intensity of the blue color indicates the relative amount of blue dye for a particular bead, and was assumed to be proportional to the activity of the catalyst on that bead; a more intensely blue colored bead indicates a more active catalyst.

With regard to an example of the present invention, about 12,500 oligopeptide-supported beads weighing a total of about 25 milligrams, corresponding to about 8.2 total micromoles of oligopeptide, were pretreated by

incubation in a room-temperature, aqueous solution of zirconium tetrachloride, $ZrCl_4$ (4.0 mg, 17 μmol in 0.6 mL water), for about 3 days. The beads were removed from solution, washed with about 10 mL of water, and air-dried. A solution of EPPS buffer (EPPS = hydroxyethyl-piperazinepropanesulfonic acid) at a pH of 5.3 was prepared. A buffered solution was prepared by combining about 0.3 mL of a 320 millimolar (mM) solution of the EPPS buffer solution with 0.3 mL of a 5.4 millimolar (mM) solution of 4-chloro-5-bromo-indolyl phosphate (about 1.6 μmol) and 0.3 mL of a 8.6 mM solution of zirconium tetrachloride (about 2.6 μmol). About 2600 of the pretreated beads, weighing 5.3 mg were incubated in the buffered solution at room temperature for about 15 h. They were removed from the buffered solution, washed with about 10 mL water, air dried, and immobilized on a tacky dot™ plate in the form of an array. Each member of the array was analyzed by X-ray fluorescence spectrometry. The tacky dot™ plate was placed in the EDAX MXRF instrument under vacuum. The instrument was operated at 35 kV and 500 μA using a rhodium X-ray tube. The area scanned was 14 x 7.4 mm using a step size of 27 μm in the x-direction and 19 μm in the y-direction. The pixel area was 512 x 400 with a 200 μsec dwell time per point. The point spectra were acquired with an acquisition time of 100 live seconds, the amount of time that the detector is actively obtaining counts. The single point spectra provided the elemental intensities for the beads, several of which are listed in the Table below.

Table.

Bead	Color	Zr (counts)	Br (counts)	Br/Zr	P (counts)
1	Yellow	93.68	303.49	3.24	545
2	Amber	34.92	118.74	3.40	82.21
3	Clear	13.75	115.2	8.38	184.41
4	Dark gray/blue	7.92	299.68	37.84	104.29
5	Yellow	37.69	330.98	8.78	264.62
6	Dark yellow	4	157.93	39.48	50.16
7	Clear	6.06	131.88	21.76	32.21

8	Gray	0	81.9	0.00	29.72
9	Dark yellow	10.27	262.58	25.57	116.86
10	Gray/blue	19.56	105.71	5.40	21.87
11	Yellow	15.56	259.2	16.66	191.59
12	Yellow	11.35	241.71	21.30	79.2
13	Blue/gray	47.93	276.95	5.78	86.24
14	Clear	15.12	178.57	11.81	185.84
15	Yellow	11.15	119.19	10.69	100.88

The most effective bead-supported catalyst produces the most product per unit of catalyst, and is a blue colored bead with a high Br count and a low P count and large Br/Zr ratio. Of the fifteen beads listed in the above Table, bead 4 includes all of these features. Blue beads 10 and 13 have significantly smaller Zr/Br ratios than bead 4, indicating poorer catalyst activity. In addition, those beads with a high Zr content bind strongly to Zr, indicating that the corresponding oligopeptides could be used as a separation agent specific for Zr. Analysis by MALDI-TOF Mass spectrometry, or by Edman degradation as previously described, can provide the amino acid sequences for the oligopeptides.

The examples described involve monitoring the elements P, Br, and S, which are present in the binder but not in any of the receptors. The X-ray fluorescence signal due to these elements indicates that the particular binder with these elements is present, making the determination of a binding effect straightforward. It should be understood that the X-ray fluorescence spectrometer can determine whether a particular element is present and also the quantity of that element. Therefore, the analysis may include a determination of the quantity of an element common to both the binder and the receptor. If a binding event occurs between a binder and receptor, and both the binder and receptor include the elements carbon and oxygen, for example, an X-ray spectrometer capable of analyzing for these elements can distinguish between the amount of these elements present in the receptor and the amount present in a binder-receptor

complex. For binders that only include elements that are also common to the receptor, the difference in the signal intensity between the receptor and the binder-receptor complex for these elements provides an indication that a binding event has occurred.

Other metal ions, purchased as standard ICP solutions (100 μg/mL in 2% HNO_3), were also tested as binders; they include ions of Be, Ca, Cd, Co, Cr, Cu, Fe, Li, Mg, Mn, Mo, Ni, Pb, Sb, Se, Sr, Ti, Tl, V, and Zn. Metal ions of Ce, Ag, Ba and Hg, available as a standard solution, 1000 μg/mL in 2 % HNO_3 solution, were also tested as potential binders.

Oligopeptides are only one type of receptor that can be used with the present invention, and clearly many other types of receptors can also be used. Esters, amines, imines, aldehydes, ketones, amides, ethers, olefins, halogenated organic molecules, antibodies, drugs, steroids, amino acids, and nucleotides can be used. Other types of oligomers such as oligonucleotides, oligosaccharides, and oligopeptides can be used. Polymers such as polyolefins, polyurethanes, polyesters, polycarbonates, polyamines, polyamides, halogenated polymers, polypeptides, polynucleotides, and polysaccharides, and nucleic acids, to name a few, can also be used as receptors. Both naturally occurring and man-made materials can be used. Also, complex structures of molecules can be used as receptors that include cell membrane receptors, viruses, cells, cellular membranes, organelles, and the like.

Similarly, many different materials can be tested as potential binders. These materials include organic molecules such as esters, amines, imines, aldehydes, ketones, amides, ethers, olefins, and halogenated organic molecules. Antibodies, drugs, hormones (e.g. steroids and the like), amino acids, and nucleic acids can also be tested as potential binders. Oligomers such as oligonucleotides, oligosaccharides, and oligopeptides, and polymers such as polyolefins, polyurethanes, polyesters, polycarbonates, polyamines, polyamides, halogenated polymers, polypeptides, polynucleotides, polysaccharides, nucleic acids, to name a few, can be also be tested as binders.

Both naturally occurring and man-made materials can be used as binders. Metal ions such as calcium ion, barium ion, sodium ion, potassium ion, iron ion, palladium ion, silver ion, and strontium ion can be used. Anions such as bromide, chloride, iodide, sulfide, and selenide, for example, can be tested as potential binders. Complex ions such as oxyanions, polyoxoanions, phosphate, organophosphates, sulfate, organosulfates, zirconate, molybdate, tungstate, chromate, for example, can be tested. Also, agonists and antagonists for cell membrane receptors, toxins, enzymes, enzyme substrates, cofactors, and monoclonal antibodies can also be tested.

The invention can also be used to determine the binding affinity (BA) between a binder and a receptor. The BA is defined as the concentration of the binder-receptor complex divided by the product of the concentrations of the binder and the receptor. To determine the binding affinity (BA), the concentrations of the binder and receptor, and the concentration of the binder-receptor complex must be determined. There are several ways, both non-destructive and destructive (e.g. removal of the receptor from the support bead), of determining the concentration of the receptor. The concentration of the receptor can be determined directly and non-destructively (i.e. the receptor remaining on the bead) by a spectroscopic technique such as by X-ray fluorescence. The receptors can also be cleaved from the bead, collected, and quantified using high performance liquid chromatography. The concentration of the receptor can also be estimated indirectly from parameters related to the support beads, such as the size and surface area of the beads.

Similarly, the concentration of the binder can be determined by quantifying the amount of binder remaining in solution after the bead-supported receptors have been submerged in the binder solution for the time period allowed for binding to occur.

It is generally assumed, particularly in the development of new catalysts on ligand (e.g. a peptide ligand) coated beads, that the distribution of ligand on each bead is similar. This often unstated assumption is illustrated by the methods used to determine catalyst efficiency. These methods as exemplified in the

aforementioned Berkessel et al. publication rely on quantifying the product of the catalytic reaction without quantifying the amount of catalyst that is formed. Product quantification methods that ignore the quantification of the catalyst lead to faulty conclusions about catalyst efficiency since catalyst efficiencies cannot be determined without knowledge of the quantity of the catalyst/catalysts used.

The present invention uses micro-X-ray fluorescence to determine the presence and relative amounts of elements. These elements can be in the form of metal ions such as calcium ion, chloride, bromide, iodide, phosphorus, and sulfur, the latter two being important constituents of polypeptides such as enzymes, RNA, and DNA. Thus, the invention provides a non-destructive method of screening the binding of a receptor array to a potential binder, and for quantifying the binding affinity. A commercially available micro-X-ray fluorescence spectrometer has been used to evaluate the binding of materials to an array of oligopeptides, each oligopeptide bound to a polystyrene bead substrate.

In summary, the present invention provides a method for detecting binding events between arrayed receptors and potential binders. The invention provides significant advantages over known methods for measuring binding affinities since known methods often require either radioactive binders, or binders that include a covalently attached label that fluoresces upon exposure to ultraviolet excitation radiation. Since the invention does not require radioactive or chemically tagged materials, the problems dealing with handling of radioactive materials and the disposal of radioactively contaminated waste are avoided. Importantly, since the use of artificially tagged materials is not required, there can be no interference from the tag in the evaluation of the binding affinity of the corresponding desired untagged material. Further, in contrast to methods that require tags, the method of the present invention can be used to evaluate the binding affinity of materials that do not fluoresce while exposed to ultraviolet radiation. It should be understood that although tagged materials are not required, they could also be used and this aspect of the invention offers a distinct advantage in that the invention can provide a direct comparison of binding affinity of the untagged binder

with that of the corresponding tagged surrogate. This comparison could validate or invalidate the assumption that a particular untagged binder and its tagged surrogate have the same binding affinity to a particular substrate.

The foregoing description of the invention has been presented for purposes of illustration and description and is not intended to be exhaustive or to limit the invention to the precise form disclosed, and obviously many modifications and variations are possible in light of the above teaching.

The embodiment(s) were chosen and described in order to best explain the principles of the invention and its practical application to thereby enable others skilled in the art to best utilize the invention in various embodiments and with various modifications as are suited to the particular use contemplated. It is intended that the scope of the invention be defined by the claims appended hereto.